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13. ABSTRACT ***(Maximum 200 Words)*** In this study, neuroprotective ganglioside derivatives are examined so they can be targeted to specific points in cell death pathways. GM1 ganglioside and several of its chemically modified derivatives are neuroprotective in several neurotoxic models. Here, ganglioside functional groups required for neuroprotection and blood-brain barrier (BBB) permeance are determined. Cell death mechanisms are also defined, as are the mechanism(s) by which ganglioside derivatives intervene in the cell death process. In the first year, substantial quantities of GM1 have been isolated and purified. LysoGM1 has been synthesized as a test substance and as starting material for the C2, C4, C8, C14, and C20 fatty acid chain length derivatives that have been synthesized and characterized. Preliminary tests on lysoGM1 and LIGA20 indicate that the ceramide fatty acid chain does not influence cytoprotection. These derivatives, however, may contribute to BBB permeance. Future studies should include delineation of MPP^+ cell death mechanisms in differentiated SH-SY5Y cells based on results that show MPP^+ (1) decreases expression of NADH:ubiquinone oxidoreductase (Complex I) subunit 4, a mitochondrial gene important for electron transport chain complex I function, and (2) increases expression of the transcriptional regulator GADD153/CHOP, a gene that is up-regulated in endoplasmic reticulum stress.

14. SUBJECT TERMS
Parkinson's disease, MPTP, MPP+, GM1, gangliosides, ganglioside derivatives, SH-SY5Y cells

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INTRODUCTION

Natural and semisynthetic gangliosides may protect neurons prior to, and following, neurotoxin exposure. The hydrophilic property of gangliosides, however, restricts their blood-brain barrier (BBB) permeability when given peripherally. This hinders their use as neuroprotective agents. Gangliosides are amenable to chemical derivatization so that semisynthetic derivatives with both cytoprotective properties and improved ability to cross the BBB can be produced. For example, gangliosides with C2, or dichloro-C2, short chain fatty acids in the ceramide moiety are more cytoprotective than the parent ganglioside GM1; and the electrochemically neutral internal ester of GM1 crosses an endothelial cell model of the BBB significantly better than its parent compound (Wells et al., 1996). This study examines ganglioside functional group derivatives that provide cytoprotection AND effectively cross the BBB; information that will provide a basis for future studies of neuroprotective mechanisms. This research studies the ability of ganglioside derivatives to be cytoprotective in *in vitro* models using the dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+) and the SH-SY5Y human neuroblastoma cell line. Derivatives determined to have therapeutic potential are tested *in vitro* for their ability to cross a brain capillary endothelial cell culture model of the BBB. Derivatives that are both cytoprotective and that effectively cross the *in vitro* BBB model will be tested *in vivo* for their ability to neuroprotect dopaminergic neurons in both chronic and acute neurotoxicity models using the MPP^+ precursor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This research studies the hypothesis that "changes in ganglioside ceramide and/or oligosaccharide functional groups will improve neuroprotection through changes in cytoprotection and BBB transcytosis." This research will provide a basis to improve ganglioside neuroprotection in neurodegenerative diseases, e.g., Parkinson's disease, and neurotoxin exposure.

BODY

This proposal consists of 4 specific objectives in the **Statement of Work.**

Statement of Work, Objective 1- Semisynthetic ganglioside derivatives will be synthesized from gangliosides isolated and characterized in the P.I.'s laboratory. These will include derivatives of the ceramide fatty acids, oligosaccharide functional groups, including internal esters, asialo, and reduced carboxylic acid (gangliosidol), and combinations of ceramide and oligosaccharide derivatives. Syntheses will be performed as described in Methods. Semisynthetic ganglioside derivatives will be characterized by chemical and mass spectrometric techniques.

In the first year, over 10 gms of GM1 have been isolated and characterized by chemical, chromatographic, and mass spectrometry techniques. The isolated GM1 has been carefully

characterized by chemical and instrumental analysis to insure purity, including the absence of peptides (Serma and Touchstone, 1974). The isolated GM1 is resorcinol and orcinol positive, yields a negative response to ninhydrin, and is retained by anion exchange resins (Ledeen and Yu, 1982). High-performance thin-layer (HPTLC) chromatography provides R_f values comparable to known GM1 standard and shows no contaminating materials on examination with ninhydrin, resorcinol, orcinol, or charring sprays. Mass spectrometry analysis provides the expected spectra for pure natural GM1 (Figure 1, Panel A).

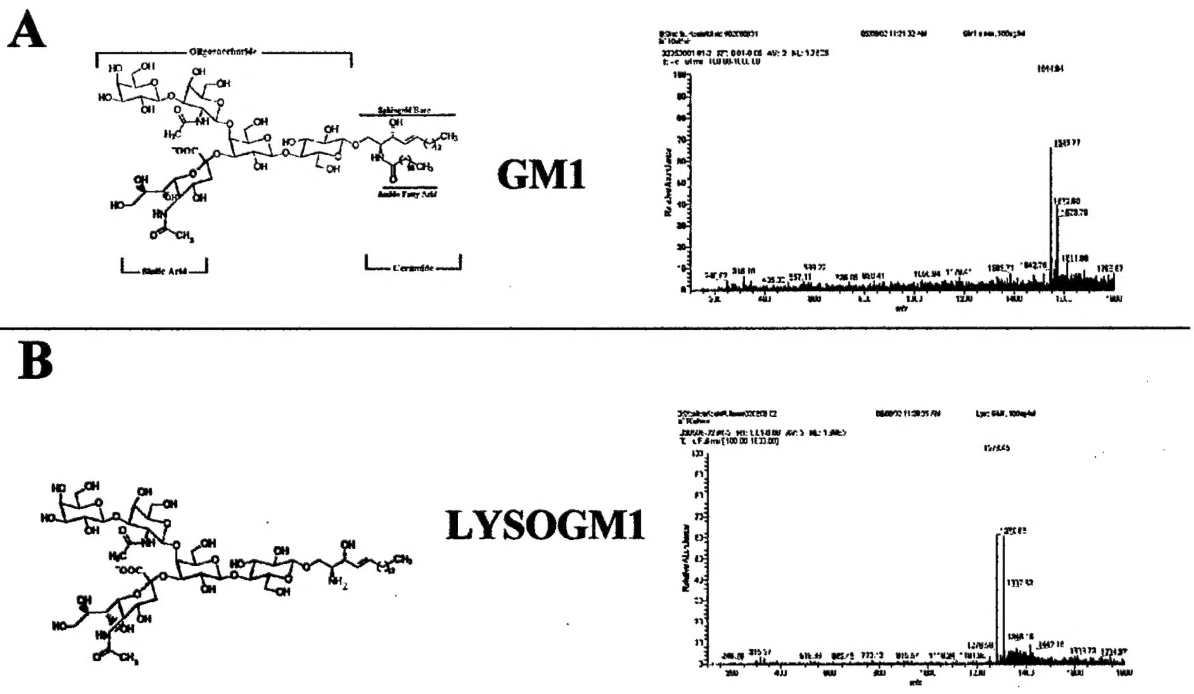

Figure 1. Structures and mass spectra of GM1 (Panel A) and lysoGM1 (Panel B): starting materials for synthesis of derivatives

LysoGM1 has been synthesized from the isolated GM1. The lysoGM1 will be tested for cytoprotective properties and used as the starting material for the synthesis of ganglioside ceramide fatty acid derivatives. It is produced by chemical reaction of GM1 with KOH in isopropanol (Sonnino et al., 1992) and characterized in the same fashion as GM1. The isolated and purified lysoGM1 has been characterized to insure purity. The material is resorcinol and orcinol positive, and yields a positive response to ninhydrin. HPTLC provides R_f values comparable to known lysoGM1 standard (Matreya Inc. State College, PA) and shows no contaminating

materials on examination with ninhydrin, resorcinol, orcinol, or charring sprays. Mass spectrometry analysis provides the expected spectra for pure lysoGM1 (Figure 1, Panel B). Other derivatives synthesized to this time include LIGA 20 (dichloroacetylGM1), and the C4, C8, C14, and C20 fatty acid semisynthetic gangliosides. Low yields of the lysoGM1 and the presence of appreciable side products from the propanolysis slowed progress slightly. Yields have increased by altering the reaction conditions. Purification of lysoGM1 and the ganglioside derivatives was performed by high-performance liquid chromatography (HPLC). Up to 5 mg of glycoconjugate are placed on a 55 cm x 7 mm I.D. Iatrobead column (mean particle size 10μm) and separated with a flow rate of 1 ml/min and a linear 140 min mobile phase gradient of 82.4% solvent A (isopropanol:hexane:water 55/38/7, v/v/v) and 17.6% solvent B (isopropanol, hexane, water, 55/20/18, v/v/v) to 51.4% solvent A and 48.6% solvent B followed by and additional 40 min. isocratic plateau at the final gradient conditions. In the case of all derivatives, purification presents the hardest challenge. For the most part, these challenges can be overcome by HPLC purification of the derivatives. Synthesis of derivatives will continue into the second year of this project.

Statement of Work, Object 2 - Semisynthetic derivatives that specifically retain or improve the cytoprotective properties of the parent compound will be determined by testing them *in vitro* using an MPP^+ model of neurotoxicity in SH-SY5Y cells. Cytoprotection will be evaluated by use of the MTT assay, trypan blue exclusion with cell counting, and neurochemical analysis of DA, DOPAC, and HVA.

Initial studies to optimize the cytotoxic model (SH-SY5Y cells with MPP+ as toxin) began in the second half of this first year. MPP+ dose response curves were determined in cultures of retinoic acid (RA) differentiated cells. An MPP+ concentration of 1 mM with two days exposure of RA-differentiated SH-SY5Y cells provides a 30% to 50% decrease in cell viability as determined by the MTT assay. These conditions are used for testing cytoprotection by ganglioside GM1 and its derivatives. Our earlier studies on MPP+-induced cell death mechanisms, utilizing undifferentiated cells, indicate that both decreased microsomal function, including decreased expression of the NADH:ubiquinone oxidoreductase (Complex I) subunits 4 and 6 ((Conn et al., 2001), Appendix Item #1) and alterations in endoplasmic reticulum, including specific regulation of GADD153/CHOP (Conn et al., 2002), Appendix Item #2) are important alterations in gene expression leading to cell death in MPP+ toxicity. It is recommended that these genes be investigated in studies in differentiated cells during the process of defining ganglioside derivative cytoprotective mechanisms. Additional studies

using gene microarray technology would also serve to guide investigations of ganglioside derivative cytoprotection.

Currently, cytoprotection of ganglioside GM1 and its derivatives are performed by plating 10,000 SH-SY5Y cells in each well of a 48-well plate with DMEM media and 10% fetal calf serum. Retinoic acid is included in the media to induce differentiation. Differentiated cells are used because they more closely represent a nondividing neuronal phenotype. After 4 days in culture the media is changed to DMEM with 0.5% serum and GM1 is added one hour prior to MPP+ addition. GM1 is again added on the second day of MPP+ exposure. Low serum content is utilized to avoid excessive ganglioside binding to serum proteins. The lower serum content does not alter cell viability as determined by the MTT assay. GM1 concentration curves revealed that 690 nM GM1 increases cell viability from 70% to approximately 90% compared to controls. The same results were obtained with lysoGM1 and LIGA20 (Figure 2). Lesser preincubation times provided less, but still significant, protection by all three compounds.

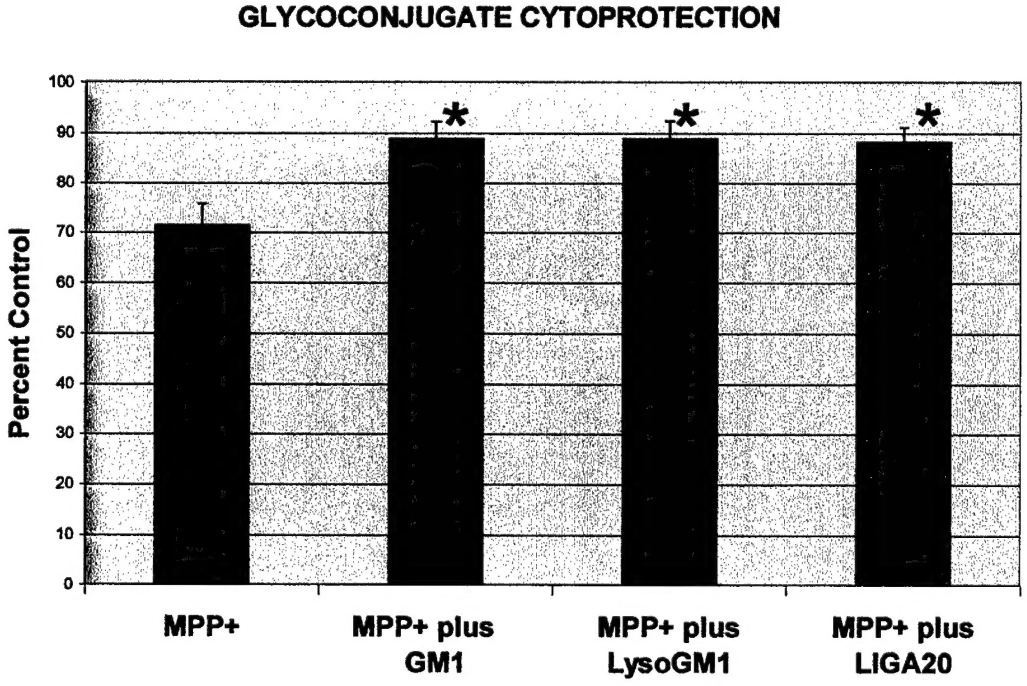

Figure 2. Cytoprotection by GM1, lysoGM1, and LIGA 20.
Values are percent control ± SEM.

* Differs from MPP+ only, One-way ANOVA, Tukey-Kramer post-hoc test, $p<0.05$

These preliminary data suggest that the ceramide fatty acid moiety may not contribute significantly to cytoprotection. More likely, the ceramide fatty acid will influence blood-brain barrier penetrance that will be determined in future studies. The glycoconjugates alone marginally increase cell viability. This experimental paradigm allows us to determine improvements in cytoprotective capacity of GM1 derivatives. With no GM1 preincubation, statistically significant cytoprotection is lost and assay variability is substantially increased. It has become apparent from the GM1 studies that it is imperative to optimize all comparisons of GM1 derivatives for preincubation time and concentration. Currently, neurochemical analysis of isolated media from cultures exposed to MPP+ and MPP+ plus GM1 or GM1 derivatives are underway.

Also, preliminary studies have been conducted with Dr. Santosh D'Mello, University of Texas, Dallas, to examine the possibility for collaboration in other models of toxicity. Early results have shown slight, but reproducible, GM1 protection in cerebellar granule cells grown under low potassium conditions.

Statement of Work, Objective 3, Effective cytoprotective semisynthetic ganglioside derivatives that effectively cross a brain capillary endothelial cell model of the blood-brain barrier (BBB) will be determined. Model BBB transcytosis will be assessed by liquid scintillation counting of radiolabeled derivatives in aliquots taken from the lower wells of Transwell cell culture plates. Although these studies are not scheduled to begin until the second half of year 2, preliminary modifications to optimize the established BBB model system are underway. Tissue resistance measurement by an EndOhm ohmmeter (World Precision Instruments, Sarasota, FL) will simplify determinations of tight junction integrity. Also, we are testing the use of track etched polyethylene terephthalate (PET) membranes (Becton-Dickinson, Bedford, MA) because they allow observation of cell growth and morphology by light microscopy on the inserts.

Statement of Work, Objective 4, Semisynthetic ganglioside derivatives that effectively protect neurons *in vivo* using chronic and acute MPTP administration models of neurotoxin insult will be determined by testing, in mice, those derivatives that both cytoprotect the SH-SY5Y cells from MPP^+ toxicity *in vitro* AND that effectively cross the *in vitro* BBB model. The chronic and acute models represent apoptotic and necrotic cell death mechanisms, respectively. Neuroprotection will be evaluated by neurochemical analysis of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), and by neuronal counts of Nissl substance, tyrosine hydroxylase (TH) and dopamine transporter (DAT) positive neurons of the substantia nigra pars compacta. Work on this objective is scheduled to begin in the second half of year two.

KEY RESEARCH ACCOMPLISHMENTS

- Isolation and purification of over 10 gm of GM1
- Synthesis of lysoGM1 as starting material and test compound
- Synthesis of GM1 fatty acid derivatives
- Established conditions for GM1 cytoprotection in retinoic acid differentiated SH-SY5Y cells

REPORTABLE OUTCOMES

Manuscripts

Conn, K. J., **Ullman, M. D.**, Eisenhauer, P., Wells, J. M., Fine, R.: Decreased expression of the NADH:ubiquinone oxidoreductase (Complex I) subunits 4 and 6 in 1-Methyl-4-phenyl-pyridinium (MPP+)-treated SH-SY5Y neuroblastoma cells, *Neuroscience Lett.* 306:145-148 (2001).

Conn KJ, Wen-Wu G, **Ullman MD**, KcKeon-O'Malley C, Eisenhauer PB, Fine RE, and Wells JM: Upregulation of GADD153/CHOP in MPP+-treated SH-SY5Y cells. *Journal of Neuroscience Research*, 68(6): 755-760 (2002)

Abstracts

Conn K.J., **Ullman M.D.**, Eisenhauer P.B., Fine R.E., Wells J.M.: Decreased Expression of the NADH:Ubiquinone Oxidoreductase (Complex I) Subunit 4 (ND4) in 1-Methyl-4-phenylpyridinium (MPP^+)-treated Human Neuroblastoma SH-SY5Y Cells. Neuroscience Meetings, San Diego, CA November (2001).

Ullman M.D. Neuroprotection by Ganglioside Derivatives. Combined Neuroscience Symposium, University of Massachusetts Medical School, Worcester, MA. June 4 (2001).

Presentations

Ullman M.D.

Location: University of Massachusetts Shriver Center

Title: "Approaches to Ganglioside Neuroprotection "

Date: March 20, 2002

Presentations (continued)

Ullman M.D.

Location: University of Massachusetts Lowell

Title: "Neuroprotective Ganglioside Derivatives"

Date: April 8, 2002

Ullman M.D.

Location: Boston University School of Medicine

Title: Neuroprotective Ganglioside Derivatives

Date: April 22, 2002

CONCLUSIONS

Our experience in synthesizing and developing HPLC procedures to purify semisynthetic ganglioside derivatives provides the opportunity to find improved cytoprotective ganglioside derivatives, to define required functional groups for cytoprotection, and to initiate studies to determine cytoprotective mechanisms. Improved cytoprotection can be examined in the SH-SY5Y model system optimized in the first year of this research. The preliminary finding that GM1, lysoGM1, and LIGA20 have essentially the same cytoprotective capacity implies that the fatty acid moiety may be of minimal importance in the cytoprotective mechanism. Most likely, however, this moiety will be important in blood-brain barrier penetrance; studies to be initiated in the second half of the second year of this research. Early analysis of gene expression in the MPP+ model indicates that both mitochondrial dysfunction and endoplasmic reticulum stress may be involved in the mechanism of MPP+ toxicity. Information of this type will be utilized to better determine possible sites of ganglioside action. It will also lay the groundwork to determine mechanisms of ganglioside derivative action through gene array technology in the absence and presence of cytoprotective compounds. Therapeutic strategies may be devised by using ganglioside derivatives that better access the brain and by understanding the functional groups required for neuroprotection. Further, by better understanding neurotoxic and neuroprotective mechanisms, specific steps in the cell death process can be targeted.

REFERENCES

Conn, K.J., Ullman, M.D., Eisenhauer, P.B., Fine, R.E. and Wells, J.M. Decreased expression of the NADH-ubiquinone oxidoreductase (Complex I) subunits 4 and 6 in 1-Methyl-4-phenyl-pyridinium (MPP+)-treated SH-SY5Y neuroblastoma cells. *Neurosci.Letts.* 306:145-148, 2001.

Conn, K.J., Wen-Wu, G., Ullman, M.D., et al. Upregulation of GADD153/CHOP in MPP+-treated SH-SY5Y cells. *J.Neurosci.Res.* 68:755-760, 2002.

Ledeen, R.W. and Yu, R.K. Gangliosides: Structure, Isolation and Analysis. *Meth.Enzymol.* 83:139-191, 1982.

Serma, J. and Touchstone, J.C. Quantitative thin layer chromatography of amino acids using fluorescamine reagent. *Analyt.Letts.* 7:279-287, 1974.

Sonnino, S., Acquotti, D., Kirschner, G., et al. Preparation of lyso-GM1 (II^3Neu5AcGgOse$_4$-long chain bases) by a one-pot reaction. *J.Lipid Res.* 33:1221-1226, 1992.

Wells, J.M., Ventura, R.F., Eisenhauer, P.B., McKenna, D.C., Fine, R.E. and Ullman, M.D. Transport of GM1 and GM1 inner ester across an in vitro model of the blood-brain barrier. *Neurosci.Letts.* 217:121-124, 1996.

APPENDICES

Journal article

Conn, K. J., Ullman M. D., Eisenhauer, P., Wells, J. M., Fine, R.: Decreased expression of the NADH:ubiquinone oxidoreductase (Complex I) subunits 4 and 6 in 1-Methyl-4-phenyl-pyridinium (MPP+)-treated SH-SY5Y neuroblastoma cells, *Neuroscience Lett.* 306:145-148 (2001).

Journal Article

Conn KJ, Wen-Wu G, Ullman MD, McKeon-O'Malley C, Eisenhauer PB, Fine RE, and Wells JM: Upregulation of GADD153/CHOP in MPP+-treated SH-SY5Y cells. *Journal of Neuroscience Research*, 68(6): 755-760 (2002).

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Neuroscience Letters 306 (2001) 145–148

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Decreased expression of the NADH:ubiquinone oxidoreductase (complex I) subunit 4 in 1-methyl-4-phenylpyridinium -treated human neuroblastoma SH-SY5Y cells

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Abstract

Oxidative stress and mitochondrial dysfunction have been implicated in Parkinson's disease (PD) pathology. NADH:ubiquinone oxidoreductase (complex I) (EC 1.6.99.3) enzyme activity is aberrant in both PD and 1-methyl-4-phenylpyridinium (MPP^+) models of PD. Reverse transcription polymerase chain reaction of RNA isolated from MPP^+-treated human neuroblastoma SH-SY5Y cells identified changes in steady-state mRNA levels of the mitochondrial transcript for subunit 4 of complex I (ND4). Expression of ND4 decreased to nearly 50% after 72 h of MPP^+ (1 mM) exposure. The expression of other mitochondrial transcripts did not change significantly under the same conditions. Pre-incubation of cells with the free-radical spin-trap, N-tert-butyl-α-(2-sulfophenyl)-nitrone prior to MPP^+ exposure, prevented decreases in cell viability and ND4 expression. This suggests that functional defects in complex I enzyme activity in PD and MPP^+ toxicity may result from changes in steady-state mRNA levels and that free radicals may be important in this process.

Keywords: Parkinson's disease; 1-Methyl-4-phenyl-pyridinium; Reverse transcription-polymerase chain reaction; Complex I; NADH:Ubiquinone Oxidoreductase subunit 4; N-tert-butyl-α-(2-sulfophenyl)-nitrone; MPP^+

Parkinson's disease (PD) is a neurodegenerative disorder with no uniformly identifiable etiology. Oxidative stress [8] and mitochondrial dysfunction [11] have been implicated in the degeneration of nigrostriatal dopaminergic neurons in PD. NADH:ubiquinone oxidoreductase (complex I) (EC 1.6.99.3) enzyme activity is decreased in PD tissue [11], PD cytoplasmic hybrids [21] and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models of PD [14].

MPTP is a potent neurotoxin that causes a parkinsonian-like syndrome in humans [12]. It has been used to produce PD models in both non-human primates and mice. The neurotoxic MPTP metabolite, 1-methyl-4-phenylpyridinium (MPP^+), is actively transported into dopaminergic neurons by dopamine transporters [7] where it is concentrated in the mitochondria [15]. There, it inhibits complex I of the electron transport chain (ETC) [14,16]. The resulting impairment of ATP generation and the associated increase in free radical production are thought to contribute to the neurotoxic action of MPP^+ [20].

MPTP and MPP^+ are neurotoxic to cells in culture. Exposure of human SH-SY5Y neuroblastoma cells to MPP^+ (1 mM for 72 h) induces production of reactive oxygen species (ROS) and apoptotic death [9]. Using this cell culture model we observe, by reverse transcription-polymerase chain reaction (RT-PCR) analyses, that MPP^+ does not significantly change the mRNA levels of several antioxidant enzymes (unpublished data) but decreases the expression of the mitochondrial encoded NADH:Ubiquinone Oxoreductase (Complex I) subunit 4 (ND4). ND4 is one of 13 polypeptides, all of which are subunits of enzyme complexes of the oxidative phosphorylation system encoded on the double-

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stranded, circular, mitochondrial DNA (mtDNA). Together with polypeptides coded by nuclear DNA, these peptides are essential to the assembly and function of the ETC complexes I, III, IV and V [22].

Expression of ND4 is essential for complex I activity [5]. It is also mutated in neuropathologically confirmed idiopathic PD [10] and familial multisystem degeneration with parkinsonism [19]. To further evaluate the MPP^+-induced decrease in ND4 steady-state mRNA, RT-PCR analyses were performed using RNA isolated from SH-SY5Y cells treated for 72 h with 0.05, 0.1, 0.5 and 1.0 mM MPP^+.

Cells were plated at a density of 1×10^6 cells per dish in 100 mm tissue culture dishes (Corning; Cambridge, MA) in 10 ml of high glucose Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin and cultured for 4 days at 37°C in a 95% air, 5% CO_2 humidified incubator. Freshly prepared MPP^+ (up to 1.0 mM) was added to the cultures and cells were incubated at 37°C for 72 h. All appropriate safety precautions were used in handling MPP^+ solutions.

72 h after toxin exposure, total RNA was isolated from untreated (control) and MPP^+-treated cells using the Tri Reagent (Sigma; St. Louis, MO). The integrity of each RNA preparation was monitored by ultra-violet visualization of ethidium bromide-stained RNA following electrophoresis on 1% agarose-formaldehyde gels. Approximately 50 μg of RNA was treated with 1 unit DNAse-I using the MessageClean kit (GenHunter Corp; Nashville, TN). DNAse-treated RNA was subjected to PCR analyses, as described below, using primers to G3PDH and ND4 to ensure that RNA preparations were free of genomic and mitochondrial DNA. DNAse-treated RNA (5 μg) was reverse transcribed using oligo(dT) primers provided in the Superscript Kit (Clonetech; Palo Alto, CA). The resultant cDNA was amplified by PCR using primers to ND4 (5′ GCTCCCTT-CCCCTACTCATC 3′ and 5′ ACATGGGCTTTAGGGA-GTCA 3′) that were designed (Primer3 program; www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and purchased (Gibco BRL; Grand Island, NY). To normalize values from untreated and toxin-treated cells, primers to the house-keeping glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; G3PDH) gene were purchased (Clonetech; Palo Alto, CA or Gibco BRL; Grand Island, NY).

PCR conditions were optimized (data not shown) for each primer set (ND4 and G3PDH-25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min). The PCR products were resolved by electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide and were visualized and quantified using a low light imaging system (4400 ChemiImager, Alpha-Innotech; San Leandro, CA). Spot densitometry values for each of the experimental PCR products were normalized to G3PDH. Values (Fig. 1) were obtained from multiple PCR reactions performed on 4 independent RNA isolations. One-way ANOVA ($\alpha = 0.05$) showed a significant difference in the main effect of MPP^+ concentration on ND4 gene expression ($F_{0.05}(3, 11) = 11.205$; $P < 0.001$). Tukey–Kramer post-hoc comparison showed that the two higher MPP^+ concentrations significantly decreased ND4 gene expression (0.5 mM, $P < 0.05$; 1.0 mM, $P < 0.01$).

To examine whether or not the MPP^+-induced decrease in ND4 steady-state mRNA was specific, or if it simply represented a general decrease in mitochondrial gene expression, primers to the mitochondrial genes Cytochrome c Oxidase subunit 1 (COX1; 5′ TCCTACTCCTGCTCGCATCT 3′ and 5′ TGGCAGGGGGTTTTATATTG 3′), and 16S rRNA (5′ CAGCCGCTATTAAAGGTTCG 3′ and 5′ TGGGTG-GGTGTGGGTATAAT 3′) in addition to a nuclear transcript for the complex I subunit, B14 [23] (5′ AACACTGTGCAC-CAATTCCA 3′ and 5′ CATGGAAGAACCGCATAACA 3′) were designed (Primer3 program; www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and purchased (Gibco BRL; Grand Island, NY) for RT-PCR analyses. cDNA was generated (as described above) from RNA isolated from untreated (control) and MPP^+-treated (1 mM for 72 h) SH-SY5Y cells.

PCR conditions were optimized (data not shown) for each primer set (COX1 and B14–25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min; 16S rRNA-20 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min) and the resultant PCR products were visualized and quantified as described above. Values (Fig. 2) were obtained from triplicate PCR reactions using cDNA generated from at least seven independent RNA isolations. One-way ANOVA revealed that exposure to MPP^+ had a significant effect on gene expression ($F_{0.05}(3, 30) = 10.73$, $P < 0.0001$). In

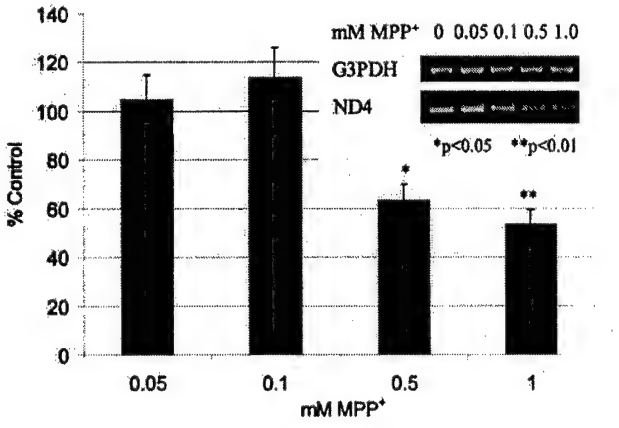

Fig. 1. Expression of ND4 in SH-SY5Y cells after exposure to different concentrations of MPP^+. RT-PCR analyses were performed using RNA isolated 72 h after MPP^+ exposure. Electrophoretic separation of PCR products from a typical experiment is shown (Insert) using primers to ND4 and G3PDH. Quantification of ND4 expression was performed as described in the text and was normalized to G3PDH values (Graph). Data are graphed as % control where control represents those cells not treated with MPP^+. Values represent the mean ± SEM of multiple PCR reactions from four independent RNA isolations.

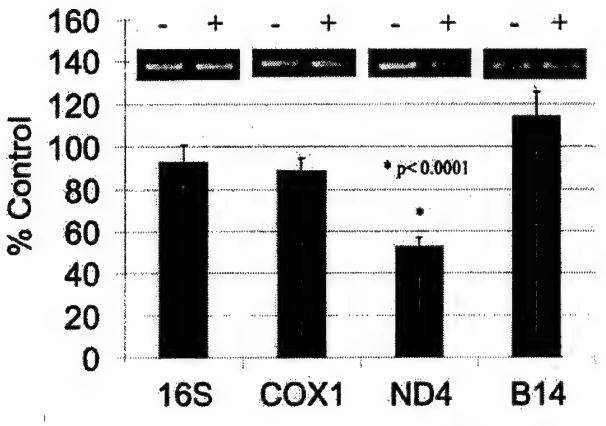

Fig. 2. Expression of 16S rRNA, COX1, ND4 and B14 in SH-SY5Y cells after exposure to MPP^+. RT-PCR analyses were performed using RNA isolated 72 h after incubation with (+) and without (−) MPP^+ (1 mM). Electrophoretic separation of PCR products from a typical experiment using primers to 16S rRNA, COX1, ND4 and B14 are shown (Insert). Quantification of ND4 expression was performed as described in the text and was normalized to G3PDH values (Graph). Data are graphed as % control where control represents those cells not treated with MPP^+. Values represent the mean ± SEM of multiple PCR reactions from at least seven independent RNA isolations.

particular, expression of ND4 was reduced significantly to nearly one half of controls (Tukey–Kramer post-hoc tests, $P < 0.05$). Expression of 16S rRNA and COX1 was slightly lower than controls following MPP^+ exposure, although this effect was not significant. B14 expression was also unaltered by exposure to MPP^+.

The observation that MPP^+ exposure decreases the expression of complex I subunit ND4 provides possible insight into the MPP^+ neurotoxic mechanism. It has been proposed that MPP^+ inhibits complex I activity because of its structural similarity to NAD^+ [14]. Because expression of ND4 is essential for complex I activity [5], this study suggests that decreased complex I activity in MPTP/MPP^+ models may arise, in part, from decreased expression of critical complex I subunits. In addition, these data support the hypothesis that changes in gene expression are important in MPP^+-mediated cell death [3,6].

It remains unclear whether MPP^+-induced reduced expression of ND4 steady-state mRNA levels results from decreased mRNA stability, reduced transcription, or both. The recent observation that hydrogen peroxide exposure decreases ND4 expression up to 4-fold in a human lens epithelial cell line suggests that free radicals may be involved in this process [2]. Exposure of human SH-SY5Y, neuroblastoma cells to 1 mM MPP^+ for 72 h induces production of ROS [9]. Thus, the role of free radicals in MPP^+-toxicity and ND4 reduced expression was examined by determining the effect of the free radical spin-trap N-tert-butyl-α-(2-sulfophenyl)-nitrone (S-PBN) on cell viability and ND4 gene expression. S-PBN attenuates striatal lesions produced by MPP^+ [18].

SH-SY5Y cells were plated at a density of 4×10^4 cells/well in 48-well tissue culture plates and grown for 4 days. They were then preincubated for 60 min with S-PBN (40 mM) and subsequently incubated with or without MPP^+ (1 mM) for 72 h as described above. The conversion of soluble 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide to insoluble formazan was used to estimate cell viability using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega; Madison, WI). S-PBN significantly improved cell viability (80.4%) in MPP^+-exposed SH-SY5Y cells compared to MPP^+-exposed cells not preincubated with S-PBN (66.6%) ($t_6 = 4.88$, $P < 0.002$).

To determine whether or not pre-incubation of cells with S-PBN affected MPP^+-mediated reduced expression of ND4 expression, RT-PCR analyses were performed in triplicate using RNA isolated on day 3 after toxin exposure from untreated (control) or MPP^+ (1 mM)-exposed SH-SY5Y cells that were pre-incubated for 60 min with or without 40 mM S-PBN. The expression of ND4 in MPP^+-treated cultures was significantly greater with S-PBN pre-incubation (93.4% of control) compared to cells exposed to MPP^+ without S-PBN pre-incubation (61.9% of control) ($t_4 = 3.42$, $P < 0.05$).

The difference in ND4 expression in S-PBN-treated cultures is likely not a reflection of increased cell viability because ND4 expression is normalized to G3PDH values between experimental conditions. Further, because S-PBN pre-incubation does not increase ND4 expression in the absence of MPP^+ (not shown), greater ND4 expression is likely not a reflection of upregulation of ND4 expression by S-PBN. Instead, the difference in ND4 expression in the S-PBN treated cultures suggests that S-PBN directly, or indirectly, blocks the reduced expression of ND4 expression by MPP^+. This may be due to the ability of S-PBN to sequester free radicals which would suggest that the production of ROS is important mechanistically in the reduced expression of ND4 expression by MPP^+.

In summary, 1 mM MPP^+ exposure decreases the expression of ND4 and the spin-trap S-PBN improves cell viability and ND4 expression after MPP^+ exposure. It will be important to examine the contribution of decreased ND4 expression to ND4 steady-state protein levels and complex I activity. Whether reduced expression of ND4 contributes mechanistically to PD neurodegeneration remains unknown. Mutations in ND4 cause the death of neurons in Leber's hereditary optic neuroretinopathy [17] and decreased ND4 expression has been measured in the affected areas of neurodegeneration in Alzheimer's disease (AD). For example, ND4 expression was decreased in AD temporal cortex [4], hippocampus and inferior parietal lobule, but not in the cerebellum [1]. ND4 expression in PD brain has not been measured. Because both AD and PD neurodegeneration is associated with the over production of free radicals, it is tempting to speculate that free radical- mediated changes in mitochondrial gene expression may contribute to the pathology of both diseases and

perhaps specificity is mediated by selected mitochondrial gene vulnerability in specific neurons. In conclusion, understanding how MPP^+ alters the expression of ND4 may provide insights into PD and possible therapeutic approaches because the neurotoxic mechanism(s) in PD and MPP^+ models share common features [13].

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[1] Aksenov, M.Y., Tucker, H.M., Nair, P., Aksenov, M.V., Butterfield, D.A., Estus, S. and Markesbery, W.R., The expression of several mitochondrial and nuclear genes encoding the subunits of electron transport chain enzyme complexes, cytochrome c oxidase, and NADH dehydrogenase, in different brain regions in Alzheimer's disease, Neurochem. Res., 24 (1999) 767–774.

[2] Carper, D.A., Sun, J.K., Iwata, T., Zigler Jr, J.S., Ibaraki, N., Lin, L.R. and Reddy, V., Oxidative stress induces differential gene expression in a human epithelial cell line, Invest. Ophthalmol. Vis. Sci., 40 (1999) 400–406.

[3] Dispasquale, B., Marini, M. and Youle, R.J., Apoptosis and DNA degradation by 1-methyl-4-phenylpyridinium in neurons, Biochem. Biophys. Res. Commun., 181 (1991) 1442–1448.

[4] Fukuyama, R., Hatanpaa, K., Rapoport, S.I. and Chandrasekaran, K., Gene expression of ND4, a subunit of complex I of oxidative phosphorylation in mitochondria, is decreased in temporal cortex of Alzheimer's disease patients, Brain Res., 713 (1996) 290–293.

[5] Hofhaus, G. and Attardi, G., Lack of assembly of mitochondrial DNA-encoded subunits of respiratory NADH dehydrogenase and loss of enzyme activity in a human cell mutant lacking the mitochondrial ND4 gene product, EMBO J., 12 (1993) 3043–3048.

[6] Itano, Y., Kitamura, Y. and Nomura, Y., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridium ion (MPP^+)-induced cell death in PC12 cells: inhibitory effects of several drugs, Neurochem. Int., 25 (1994) 419–424.

[7] Javitch, J.A., D'Amato, R.J., Strittmatter, S.M. and Snyder, S.H., Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity, Proc. Natl. Acad. Sci. USA, 82 (1985) 2173–2177.

[8] Jenner, P. and Olanow, C.W., Oxidative stress and the pathogenesis of Parkinson's disease, Neurology, 47 (1996) S161–S170.

[9] Kitamura, Y., Kosaka, T., Kakimura, J.-I., Matsuoka, Y., Kohno, Y., Kohno, Y., Nomura, Y. and Taniguchi, T., Protective effects of the antiparkinsonian drugs talipexole and pramipexole against 1-methyl-4-phenylpyridinium-induced apoptotic death in human neuroblastoma SH-SH5Y cells, Mol. Pharmacol., 54 (1998) 1046–1054.

[10] Kösel, S., Grasbon-Frodl, E.M., Mautsch, U., Egensperger, R., von Eitzen, U., Frishman, D., Hofmann, S., Gerbitz, K.D., Mehraein, P. and Graeber, M.B., Novel mutations of mitochondrial complex I in pathologically proven Parkinson disease, Neurogenetics, 3 (1998) 197–204.

[11] Kösel, S., Hofaus, G., Maassen, A., Vieregge, P. and Graeber, M.B., Role of mitochondria in Parkinson disease, Biol. Chem., 380 (1999) 865–870.

[12] Langston, J.W., Ballard, P., Tetrud, J.W. and Irwin, I., Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis, Science, 219 (1983) 979–980.

[13] Langston, J.W., Forno, L.S., Tetrud, J., Reeves, A.G., Kaplan, J.A. and Karluk, D., Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure, Ann. Neurol., 46 (1999) 598–605.

[14] Mizuno, y., Ikebe, S.-I., Hattor, N., Mochizuki, H., Nakagawa-Hattri, Y. and Kondo, T., Etiology of Parkinson's disease, In R.L. Watts and W.C. Koller (Eds.), Movement Disorders, Neurologic Principles and Practice, McGraw Hill, New York, 1997, pp. 161–182.

[15] Ramsay, R.R., Dadgar, J., Trevor, A. and Singer, T.P., Energy-driven uptake of N-methyl-4-phenylpyridine by brain mitochondria mediates the neurotoxicity of MPTP, Life Sci., 39 (1986) 581–588.

[16] Ramsay, R.R., Salach, J.I., Dadgar, J. and Singer, T.P., Inhibition of mitochondrial NADH dehydrogenase by pyridine derivatives and its possible relation to experimental and idiopathic Parkinsonism, Biochem. Biophys. Res. Commun., 135 (1986) 269–275.

[17] Savontaus, M.-L., mtDNA mutations in Leber's hereditary optic neuropathy, Biochim. Biophys. Acta, 1271 (1995) 261–263.

[18] Schulz, J.B., Henshaw, D.R., Matthews, R.T. and Beal, M.F., Coenzyme Q10 and nicotinamide and a free radical spin trap protect against MPTP neurotoxicity, Exp. Neurol., 132 (1995) 279–283.

[19] Simon, D.K., Pulst, S.M., Sutton, J.P., Browne, S.E., Beal, M.F. and Johns, D.R., Familial multisystem degeneration with parkinsonism associated with the 11778 mitochondrial mutation, Neurology, 53 (1999) 1787–1793.

[20] Shoffner, J.M., Oxidative phosphorylation diseases and movement disorders, In R.L. Watts and W.C. Koller (Eds.), Movement Disorders, Neurologic Principles and Practice, McGraw Hill, New York, 1997, pp. 51–71.

[21] Swerdlow, R.H., Parks, J.K., Miller, S.W., Tuttle, J.B., Trimmer, P.A., Sheehan, J.P., Bennett Jr, J.P., Davis, R.E. and Parker Jr, W.D., Origin and functional consequences of the complex I defect in Parkinson's disease, Ann. Neurol., 40 (1996) 663–671.

[22] Taanman, J.-W., The mitochondrial genome: structure, transcription, translation and replication, Biochim. Biophys. Acta, 1410 (1999) 103–123.

[23] Ton, C., Hwang, D.M., Dempsey, A.A. and Liew, C.C., Identification and primary structures of five human NADH-ubiquinone oxidoreductase subunits, Biochem. Biophys. Res. Commun., 241 (1997) 589–594.

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Specific Up-regulation of GADD153/CHOP in 1-Methyl-4-Phenyl-Pyridinium-Treated SH-SY5Y Cells

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Growth arrest DNA damage-inducible 153 (GADD153) expression was increased in 1-methyl-4-phenyl-pyridinium (MPP^+)-treated human SH-SY5Y neuroblastoma cells as determined by gene microarray analysis. GADD153 expression increased after 24 hr of MPP^+ (1 mM) exposure and preceded activation of caspase 3. Comparison of GADD153 expression among cultures treated with other toxins whose primary mode of action is either via mitochondrial impairment (rotenone) or via oxidative stress (6-hydroxydopamine or hydrogen peroxide) showed that GADD153 was uniquely up-regulated by MPP^+. Together these data suggest that a cellular mechanism distinct from mitochondrial impairment or oxidative stress contributes significantly to the up-regulation of GADD153 by MPP^+ and that GADD153 may function as an inducer of apoptosis following MPP^+ exposure. **Published 2002 Wiley-Liss, Inc.†**

Key words: 1-methyl-4-phenyl-pyridinium; Parkinson's disease; gene expression; apoptosis; endoplasmic reticulum

Parkinson's disease (PD) is a slow and progressive neurodegenerative disorder with no uniformly identifiable etiology. Pathological hallmarks of the disease include the death of dopaminergic neurons of the substantia nigra and the presence of Lewy bodies in the surviving neurons. Several biochemical mechanisms have been suggested for PD pathology, including mitochondrial dysfunction (Kosel et al., 1999), oxidative stress (Dexter et al., 1989; Jenner and Olanow 1996), and apoptosis (Andersen, 2001).

Neurodegeneration of dopamine-producing cells has been experimentally modeled using the toxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), rotenone, and hydrogen peroxide (H_2O_2). MPTP is a potent neurotoxin that was first recognized to cause a parkinsonian-like syndrome in humans after self-administration (Langston et al., 1983). Since then, MPTP has been used to produce PD models in both nonhuman primates and mice. The neurotoxic MPTP metabolite, 1-methyl-4-phenyl-pyridinium (MPP^+), is actively transported into dopaminergic neurons by dopamine transporters (Javitch et al., 1985), where it is concentrated in the mitochondria (Ramsay et al., 1986a). There, it inhibits complex I of the electron transport chain (ETC; Ramsay et al., 1986b). The impairment of ATP generation results in dysregulated calcium homeostasis (Mizuno et al., 1997), mitochondrial membrane depolarization (Mizuno et al., 1997), free radical production (Chance et al., 1979; Hasegawa et al., 1990), and ultrastructural changes of the endoplasmic reticulum (ER; Tanaka et al., 1988; Adams et al., 1989; Poli et al., 1990; Rapisardi et al., 1990; Mizukawa et al., 1990; Sheehan et al., 1997). MPP^+ is neurotoxic to cells in culture. Exposure of human SH-SY5Y neuroblastoma cells to 1 mM MPP^+ for 3 days induces apoptotic death as evidenced by caspase 3 activation (Kitamura et al., 1998).

As with MPP^+, rotenone is a dopaminergic toxin that causes mitochondrial dysfunction. Chronic, systemic inhibition of complex I by rotenone in rats causes highly selective nigrostriatal dopaminergic degeneration that is associated behaviorally with hypokinesia and rigidity and

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the accumulation of fibrillar cytoplasmic inclusions that contain ubiquitin and α-synuclein (Betarbet et al., 2000). The primary mechanism of cell death for both 6-OHDA and H_2O_2 is oxidative stress. H_2O_2 is produced along with superoxide and hydroxyl radicals during the autooxidation of 6-OHDA after uptake via the high-affinity dopamine uptake system (Cohen and Heikkila, 1974).

Growth arrest DNA damage-inducible 153 (GADD153), also called C/EBP homology protein (CHOP), is a basic region leucine zipper transcription factor and heterodimerizes with members of the C/EBP family of transcription factors (Ron and Habener, 1992). GADD153 was first identified as a transcript induced in response to growth arrest and DNA damage (Fornace et al., 1988). GADD153 expression is also induced by many additional cellular stresses (Schmitt-Ney and Habener, 2000, and references within). GADD153 has been proposed to play a role in several different scenarios, including growth arrest (Barone et al., 1994), apoptosis (Matsumoto et al., 1996; Igase et al., 2001; Maytin et al., 2001), and the ER stress response (Zinszner et al., 1998; Ubeda and Habener, 2000; McCullough et al., 2001).

This study demonstrates that exposure of human SH-SY5Y neuroblastoma cells to MPP^+ increases GADD153 expression and that this up-regulation precedes the activation of caspase 3. Comparison of GADD153 steady-state mRNA levels in parallel cultures treated with toxins whose primary mode of action is either via mitochondrial impairment (rotenone) or via oxidative stress (6-OHDA or H_2O_2) suggests that MPP^+-induced mitochondrial dysfunction and oxidative stress are not directly involved in the MPP^+-induced up-regulation of GADD153. Together these data suggest that a cellular mechanism distinct from mitochondrial impairment or oxidative stress contributes significantly to the up-regulation of GADD153 by MPP^+ and that GADD153 may function as an inducer of apoptosis following MPP^+ exposure.

MATERIALS AND METHODS

Materials

MPP^+ iodide, 6-OHDA, rotenone, Dulbecco's modified Eagle's Medium (DMEM; Sigma-Aldrich; St. Louis, MO), H_2O_2 (Fisher Scientific; Rochester, NY), and fetal bovine serum (FBS; Gibco BRL, Grand Island, NY) were purchased from commercial sources.

Cell Culture and MPP^+ Treatment

The human neuroblastoma cell line SH-SY5Y (ATCC CRL-2266) was cultured at 37°C in a 95% air, 5% CO_2 humidified incubator and maintained in DMEM-high glucose supplemented with 10% FBS. Cells were routinely subcultured when confluent, and the culture medium was changed twice per week. For toxin experiments, 0.5×10^6 cells were plated into 100 mm^2 dishes (Corning, Cambridge, MA) in 10 ml DMEM plus 10% FBS and 100 units/ml penicillin and 100 mg/ml streptomycin and cultured for 4 days. Freshly prepared toxins were added to the cultures and incubated at 37°C for various lengths of time. All appropriate safety precautions were used in handling toxin solutions.

RNA Isolation and Reverse Transcription

Total RNA was isolated using Tri Reagent (Sigma). The integrity of each RNA preparation was monitored by ultraviolet visualization of ethidium bromide-stained RNA following electrophoresis on 1% agarose-formaldehyde gels. Approximately 50 μg of RNA was treated with 1 unit DNAse-I using the MessageClean kit (GenHunter Corp., Nashville, TN). DNAse-treated RNA was subjected to polymerase chain reaction (PCR) analyses, as described below, using primers to G3PDH to ensure that each RNA preparation was free of DNA. DNAse-treated RNA (5 μg) was reverse transcribed using oligo(dT) primers provided in the Superscript kit (Clonetech, Palo Alto, CA).

Microarray Analysis

After 72 hr, 15×10^6 cells were collected after trypsinization from control and MPP^+ (1 mM)-treated SH-SY5Y cells. Total RNA was isolated, and radioactive ^{33}P was incorporated into cDNA in a reverse transcription reaction using gene-specific primer sequences (Clontech). Radiolabeled cDNA was hybridized with human toxicology 1.2 array membranes (Clontech), and differential gene expression was visualized by exposure to phosphoimaging cassettes. Results were analyzed using Atlas Image software (Clontech).

PCR Amplification, Visualization, and Quantification

PCR was performed as described previously (Conn et al., 2001). Primers for the amplification of the glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; G3PDH) and GADD153 cDNA were purchased (Clonetech or Gibco BRL). Conditions for G3PDH amplification were identical to those previously described (Conn et al., 2001). For the GADD153 primer set, each reaction cycle consisted of the following steps: 94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min. GADD153 reactions were carried out for 30 cycles. After resolution by electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide, PCR products were visualized and quantified using the 4400 ChemiImager low-light imaging system (Alpha-Innotech, San Leandro, CA). GADD153 expression was expressed as a ratio to the value of G3PDH product obtained from parallel reactions.

Western Blot Analysis

Cells were harvested from 100 mm^2 dishes in 300 μl of phosphate-buffered saline (PBS) using a plastic cell lifter. Harvested cells were pelleted by centrifugation (12,000*g* at 4°C), resuspended in 100 μl lysis buffer (20 mM Tris pH 7.4, 140 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1× protease inhibitor cocktail; Roche; Mannheim, Germany), and frozen to −70°C. Thawed cell suspensions were clarified by centrifugation (12,000*g* at 4°C), and the protein concentrations of the resultant supernatants were determined using the BCA kit (Pierce, Rockford, IL). Fifty micrograms of protein were resolved on a 4–12% NuPAGE gel and transferred to nitrocellulose (Invitrogen, Carlsbad, CA). After blocking membranes in 15 ml of SuperBlock buffer (Pierce) overnight at 4°C, membranes were washed for 5 min six times in 20 ml of wash buffer

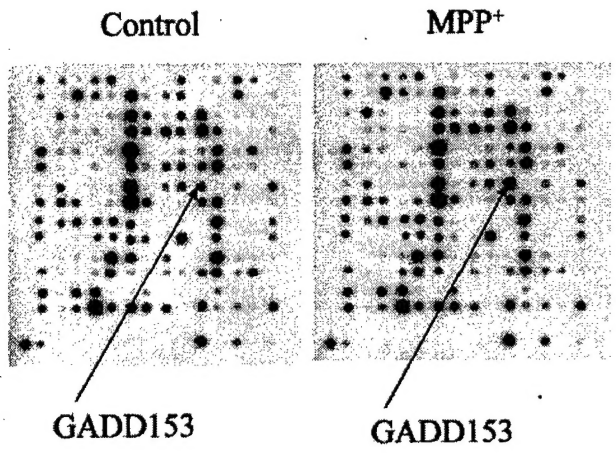

Fig. 1. Phosphoimages from control and MPP^+ membranes. Shown are phosphoimages of a section of gene microarray membrane hybridized with radiolabeled cDNA generated from RNA isolated 72 hr after exposure to vehicle (control) or MPP^+. The arrows show differential gene expression of GADD153.

[BupH buffer (Pierce) with 0.05% Tween 20]. Membranes were incubated with a 1:1,000 dilution of polyclonal rabbit anticaspase-3 (BD Bioscience, Franklin Lakes, NJ) or a 1:50 dilution of monoclonal mouse anti-GADD153 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 20 ml wash buffer for 2 hr at room temperature. Membranes were washed as described above and incubated with a 1:100,000 dilution of goat anti-rabbit horseradish peroxidase (HRP)-conjugated or rabbit anti-mouse HRP-conjugated secondary antibody (Chemicon International Inc., Temecula, CA) diluted in wash buffer for 45 min at room temperature. Membranes were washed as described above and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce). Membranes were visualized with the 4400 ChemiImager low-light imaging system (Alpha-Innotech). Exposure times were optimized to collect images of protein products within the linear range of detection for the ChemiImaging system.

RESULTS

Mitochondrial dysfunction accompanying MPP^+ toxicity may be controlled, in part, by changes in gene expression (Conn et al., 2001). Using these same experimental conditions, we have employed gene microarray analysis to generate a gene expression profile of MPP^+ toxicity at 72 hr after exposure. Phosphoimages of a membrane section hybridized with radiolabeled control and MPP^+ cDNA are shown in Figure 1. In total, 1,185 genes were assayed for MPP^+-induced changes in expression levels (positive or negative) induced by MPP^+. By using cutoff criteria recommended by the manufacturer (a threshold of threefold difference in signal and a ratio of 1.67 differential expression after normalization using a global method), 313 genes were identified as differentially expressed. The GADD153 gene showed the greatest change (Fig. 1).

A time course experiment was performed to determine whether up-regulation of GADD153 following MPP^+ exposure preceded apoptosis. The activation of apoptosis was evaluated by Western blot analysis using antibodies to caspase 3. Generation of the active form of caspase 3 by proteolysis of the pro- isoform is indicative of neuronal apoptosis (Porter and Janicke 1999). SH-SY5Y cells were treated with and without 1 mM MPP^+ for 5, 24, 48, 72, and 96 hr. Cells at each time point were isolated in parallel for RT-PCR and Western blot analysis. Equal concentrations of RNA from each experimental condition were reverse transcribed into cDNA and amplified by PCR (Fig. 2A). Equal concentrations of protein from each experimental condition were electrophoretically separated and transferred to nitrocellulose before being probed with antibodies to GADD153 (Fig. 2B) and caspase 3 (Fig. 2C).

The spot densitometry values of each PCR and protein product from two independent time course experiments were averaged and expressed as percentage control, where control represents those cells not treated with MPP^+ (Fig. 2D). After 1 mM MPP^+ exposure, GADD153 steady-state mRNA increased linearly up to 72 hr. By 96 hr, GADD153 steady-state mRNA levels had decreased significantly from that at 72 hr (two-tailed Student's t-test, $P < 0.02$). GADD153 steady-state protein levels increased linearly up to 24 hr. Caspase 3 activation increased linearly from 24 to 72 hr.

The specificity of the GADD153 response was examined in SH-SY5Y cells treated with other toxins. RT-PCR analyses were performed using RNA isolated from cells treated with the LD_{50} values for MPP^+ (1 mM), 6-OHDA (25 μM), H_2O_2 (600 μM), and rotenone (50 nM) for 72 hr. Equal concentrations of RNA from each toxin exposure were reverse transcribed into cDNA and amplified by PCR. Spot densitometry values of the PCR products were expressed as a ratio to the value of G3PDH product obtained from parallel reactions (Fig. 3). One-way ANOVA indicated that toxins had a significant effect on GADD153 expression [$F_{0.05}(4,19) = 71.587$, $P < 0.0001$]. Specifically, MPP^+ caused a threefold increase in expression (Dunnett multiple comparisons test, $P < 0.01$). GADD153 expression was essentially unaffected by 6-OHDA, H_2O_2, or rotenone. RT-PCR analyses performed using RNA isolated 5, 24, and 96 hr after 6-OHDA (25 μM), H_2O_2 (600 μM), and rotenone (50 nM) exposure also did not show any significant differences in GADD153 gene expression (data not shown).

DISCUSSION

The generation of a gene expression profile of MPP^+ toxicity may make it possible to determine the molecular mechanisms involved in MPP^+ toxicity and PD neurodegeneration. By using a gene microarray approach, this study identified GADD153 as a putative gene up-regulated by MPP^+ in a human dopaminergic neuroblastoma cell line. RT-PCR and Western blot analysis confirmed that both GADD153 steady-state mRNA and protein are up-regulated in response to MPP^+ and that

GADD153 up-regulation in MPP^+-treated cells precedes caspase 3 activation. Together these data suggest that increases in GADD153 expression may contribute to the activation of apoptosis following MPP^+ exposure. Support for this hypothesis comes from other reports showing that overexpression of GADD153 induces apoptosis in M1 myeloblastic leukemia cells (Matsumoto et al., 1996), vascular smooth muscle cells (Igase et al., 2001), 3T3 fibroblasts, keratinocytes, and HeLa cells (Maytin et al., 2001).

Elevated GADD153 expression may contribute to the activation of apoptosis by functioning as a transcriptional regulator. For example, GADD153 binds C/EBP transcription factors (Ron and Habener, 1992). Dimerization of GADD153 with C/EBP proteins inhibits transcription through C/EBP binding elements (Ron and Habener, 1992) but activates transcription via unique GADD153-C/EBP elements (Ubeda et al., 1996). GADD153 has also been shown to augment AP-1-

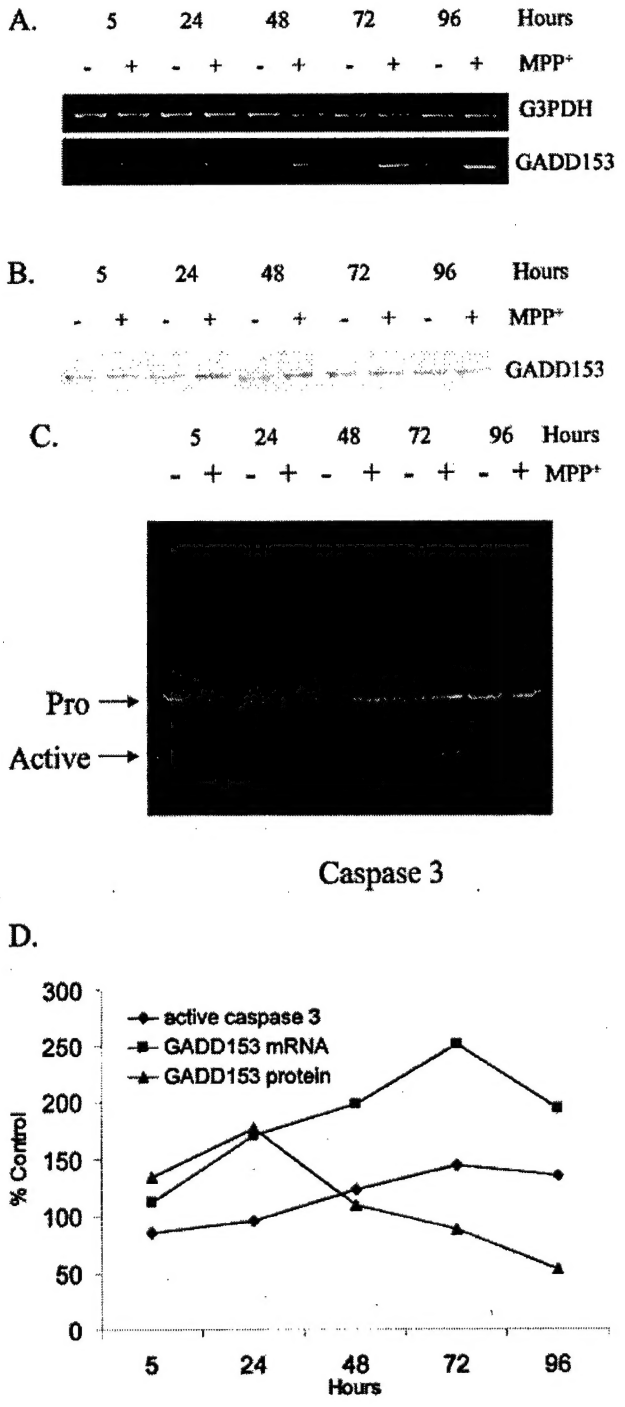

Fig. 2. Time course of GADD153 expression and caspase 3 activation in SH-SY5Y cells after exposure to 1 mM MPP^+. **A:** RT-PCR analyses were performed using RNA isolated 5, 24, 48, 72, and 96 hr after MPP^+ exposure. Electrophoretic separation of PCR products from a typical experiment is shown using primers to GADD153 and G3PDH. **B:** Western blot analyses were performed using cell extracts isolated 5, 24, 48, 72, and 96 hr after MPP^+ exposure. Extracts from a typical experiment were electrophoretically separated and transferred to nitrocellulose prior to being probed with antibodies to GADD153. **C:** Western blot analyses were performed using cell extracts isolated 5, 24, 48, 72, and 96 hr after MPP^+ exposure. Extracts from a typical experiment were electrophoretically separated and transferred to nitrocellulose prior to being probed with antibodies to caspase 3. **D:** Quantification of GADD153 steady-state mRNA, GADD153 protein, and active caspase 3 was performed as described in the text. Data are graphed as percentage control, where control represents those cells not treated with MPP^+. Values represent the mean of two independent experiments.

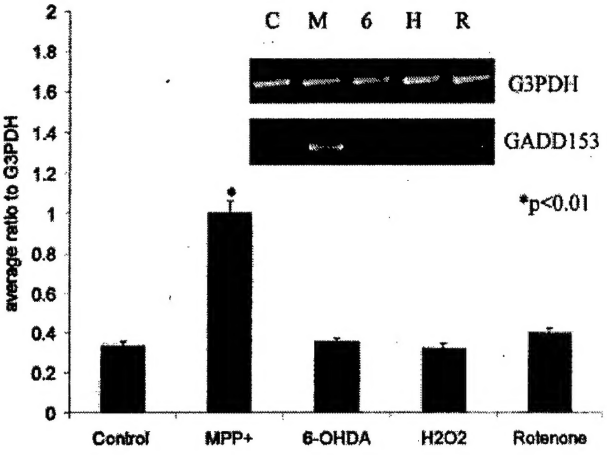

Fig. 3. Expression of GADD153 in SH-SY5Y cells after exposure to MPP^+, 6-OHDA, H_2O_2, and rotenone. RT-PCR analyses were performed using RNA isolated 72 hr after exposure to the LD_{50} concentrations of MPP^+ (M), 6-OHDA (6), H_2O_2 (H), and rotenone (R). Electrophoretic separation of PCR products from a typical experiment is shown (**inset**) using primers to GADD153 and G3PDH. Quantification of GADD153 expression was performed as described in the text and was normalized to G3PDH values (Graph). Values represent the mean ± SEM of quadruplicate RT-PCRs.

mediated transcription (Ubeda et al., 1999). Alternatively, it is possible that GADD153 could activate apoptosis through a nontranscriptional mechanism. For example, GADD153 has been shown to bind the ribosomal protein FTE/3a, resulting in the induction of apoptosis (Cui et al., 2000).

To gain insight into the type of cellular stress inducing GADD153 expression after MPP^+ exposure, we compared GADD153 expression in parallel cultures treated with toxins whose primary mode of action is either via mitochondrial impairment (rotenone) or via oxidative stress (6-OHDA or H_2O_2). The observation that none of these toxins increased GADD153 expression suggests that a cellular mechanism different from mitochondrial impairment or oxidative stress contributes significantly to the up-regulation of GADD153 by MPP^+.

One possible mechanism is that ER stress may play a role in the up-regulation of GADD153 following MPP^+ exposure. MPP^+ causes ultrastructural changes to the ER in mice (Adams et al., 1989), monkeys (Tanaka et al., 1988), dogs (Rapisardi et al., 1990), and SH-SY5Y cells (Sheehan et al., 1997). ER stress up-regulates the expression of GADD153 (Wang et al., 1996; Ubeda and Habener, 2000), and ER stress may play a role in PD dopaminergic neurodegeneration (Imai et al., 2000, 2001).

Stresses to the ER, such as disruption of calcium homeostasis, inhibition of protein glycosylation, and reduction of disulfide bonds, provoke the accumulation of unfolded proteins in the ER lumen. This pathological situation induces the activation of three highly conserved stress responses, the ER overload response (EOR), the unfolded protein response (UPR), and the ER-associated degradation pathway (ERAD; Kaufman, 1999; Pahl, 1999). The UPR is characterized by induction of gene expression of ER-localized protein-folding catalysts and protein chaperones and by the inhibition of protein translation by phosphorylation of the eukaryotic initiation factor-2α (eIF-2α). Support for the induction of the UPR by MPP^+ comes from recent RT-PCR data showing that, under conditions identical to those described in this paper, MPP^+ increases the expression of at least two ER stress genes, protein disulfide isomerase and calreticulin (Conn et al., unpublished data). If MPP^+ is inducing a UPR, this may provide insight into the observation (Fig. 2) that GADD153 steady-state mRNA levels increase linearly to 72 hr but that GADD153 protein levels increase linearly to only 24 hr. One possibility is that translation of GADD153 mRNA may be inhibited after 24 hr as a result of the phosphorylation of eIF-2α.

Disruption of calcium homeostasis may be a mechanism by which MPP^+ exposure leads to ER stress. This is unlikely to be due to inhibition of ATP-dependent ER Ca^{2+} pumps, because rotenone also inhibits ATP production but does not cause GADD153 up-regulation. Because MPP^+ has also been shown to inhibit α-ketoglutarate dehydrogenase enzyme activity (Mizuno et al., 1987), the possibility that MPP^+ decreases intracellular ATP levels to a greater extent than rotenone cannot be excluded. Alternatively, MPP^+ may cause ER stress by direct ER uptake and inhibition of enzymes responsible for normal ER function. Receptors for both vesicular monoamine transporter 2 (VMAT2; Nirenberg et al., 1995) and the dopamine transporter (DAT; Kadota et al., 1996; Nirenberg et al., 1996; Hersch et al., 1997) have been localized to the ER, supporting the possibility that MPP^+ is concentrated within the ER.

In conclusion, exposure of SH-SY5Y cells to MPP^+ increases the expression of GADD153 through a cellular mechanism that appears to be distinct from mitochondrial impairment or oxidative stress. Increased GADD153 expression precedes caspase 3 activation and, therefore, may represent an early event in the activation of the apoptotic death pathway during MPP^+ toxicity and PD neurodegeneration.

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REFERENCES

Adams JD Jr, Kalivas PW, Miller CA. 1989. The acute histopathology of MPTP in the mouse CNS. Brain Res Bull 23:1–17.

Andersen JK. 2001. Does neuronal loss in Parkinson's disease involve programmed cell death? Bioessays 23:640–646.

Barone MV, Crozat A, Tabaee A, Philipson L, Ron D. 1994. CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G1/S arrest. Genes Dev 8:453–464.

Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. 2000. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 3:1301–1306.

Chance B, Sies H, Boveris A. 1979. Hydrogen peroxide metabolism in mammalian organs. Physiol Rev 59:527–605.

Cohen G. Heikkila RE. 1974. The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. J Biol Chem 249:2447–2452.

Conn KJ, Ullman MD, Eisenhauer PB, Fine RE, Wells JM. 2001. Decreased expression of the NADH:ubiquinone oxidoreductase (complex I) subunit 4 (ND4) in 1-methyl-4-phenylpyridinium (MPP^+)-treated human neuroblastoma SH-SY5Y cells. Neurosci Lett 306:145–148.

Cui K, Coutts M, Stahl J, Sytkowski AJ. 2000. Novel interaction between the transcription factor CHOP (GADD153) and the ribosomal protein FTE/S3a modulates erythropoiesis. J Biol Chem 275:7591–7596.

Dexter DT, Carter CJ, Wells FR, Javoy-Agid F, Agid Y, Lees A, Jenner P, Marsden CD. 1989. Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. J Neurochem 52:381–389.

Fornace AJ Jr, Alamo I Jr, Hollander MC. 1988. DNA damage-inducible transcripts in mammalian cells. Proc Natl Acad Sci USA 85:8800–8804.

Hasegawa E, Takeshige K, Oishi T, Murai Y, Minakami S. 1990. 1-Methyl-4-phenylpyridinium (MPP^+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. Biochem Biophys Res Commun 170:1049–1055.

Hersch SM, Yi H, Heilman CJ, Edwards RH, Levey AI. 1997. Subcellular localization and molecular topology of the dopamine transporter in the striatum and substantia nigra. J Comp Neurol 388:211–227.

Igase M, Okura T, Nakamura M, Takata Y, Kitami Y, Hiwada K. 2001. Role of GADD153 (growth arrest- and DNA damage-inducible gene 153) in vascular smooth muscle cell apoptosis. Clin Sci 100:275–281.

Imai Y, Soda M, Takahashi R. 2000. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J Biol Chem 275:35661–35664.

Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. 2001. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell 105:891–902.

Javitch JA, D'Amato RJ, Strittmatter SM, Snyder SH. 1985. Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. Proc Natl Acad Sci USA 82:2173–2177.

Jenner P, Olanow CW. 1996. Oxidative stress and the pathogenesis of Parkinson's disease. Neurology 47:S161–S170.

Kadota T, Yamaai T, Saito Y, Akita Y, Kawashima S, Moroi K, Inagaki N, Kadota K. 1996. Expression of dopamine transporter at the tips of growing neurites of PC12 cells. J Histochem Cytochem 44:989–996.

Kaufman RJ. 1999. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 13:1211–1233.

Kitamura Y, Kosaka T, Kakimura JI, Matsuoka Y, Kohno Y, Nomura Y, Taniguchi T. 1998. Protective effects of the antiparkinsonian drugs talipexole and pramipexole against 1-methyl-4-phenylpyridinium-induced apoptotic death in human neuroblastoma SH-SY5Y cells. Mol Pharmacol 54:1046–1054.

Kosel S, Hofhaus G, Maassen A, Vieregge P, Graeber MB. 1999. Role of mitochondria in Parkinson disease. Biol Chem 380:865–870.

Langston JW, Ballard P, Tetrud JW, Irwin I. 1983. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219:979–980.

Matsumoto M, Minami M, Takeda K, Sakao Y, Akira S. 1996. Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. FEBS Lett 395:143–147.

Maytin EV, Ubeda M, Lin JC, Habener JF. 2001. Stress-inducible transcription factor CHOP/gadd153 induces apoptosis in mammalian cells via p38 kinase-dependent and -independent mechanisms. Exp Cell Res 267: 193–204.

McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. 2001. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21:1249–1259.

Mizukawa K, Sora YH, Ogawa N. 1990. Ultrastructural changes of the substantia nigra, ventral tegmental area and striatum in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. Res Commun Chem Pathol Pharmacol 67:307–320.

Mizuno Y, Saitoh T, Sone N. 1987. Inhibition of mitochondrial alpha-ketoglutarate dehydrogenase by 1-methyl-4-phenylpyridinium ion. Biochem Biophys Res Commun 143:971–976.

Mizuno Y, Ikebe S-I, Hattori N, Mochizuki H, Nakagawa-Hatori Y, Kondo T. 1997. Etiology of Parkinson's disease. In: Watts RL, Koller WC, editors. Movement disorders neurologic principles and practice. New York: McGraw-Hill. p 161–182.

Nirenberg MJ, Liu Y, Peter D, Edwards RH, Pickel VM. 1995. The vesicular monoamine transporter 2 is present in small synaptic vesicles and preferentially localizes to large dense core vesicles in rat solitary tract nuclei. Proc Natl Acad Sci USA 92:8773–8777.

Nirenberg MJ, Vaughan RA, Uhl GR, Kuhar MJ, Pickel VM. 1996. The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. J Neurosci 16:436–447.

Pahl HL. 1999. Signal transduction from the endoplasmic reticulum to the cell nucleus. Physiol Rev 79:683–701.

Poli A, Guarnieri T, Facchinetti F, Villani L. 1990. Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in goldfish brain. Brain Res 534:45–50.

Porter AG, Janicke RU. 1999. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6:99–104.

Ramsay RR, Dadgar J, Trevor A, Singer TP. 1986a. Energy-driven uptake of N-methyl-4-phenylpyridine by brain mitochondria mediates the neurotoxicity of MPTP. Life Sci 39:581–588.

Ramsay RR, Salach JI, Dadgar J, Singer TP. 1986b. Inhibition of mitochondrial NADH dehydrogenase by pyridine derivatives and its possible relation to experimental and idiopathic parkinsonism. Biochem Biophys Res Commun 135:269–275.

Rapisardi SC, Warrington VO, Wilson JS. 1990. Effects of MPTP on the fine structure of neurons in substantia nigra of dogs. Brain Res 512:147–154.

Ron D, Habener JF. 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev 6:439–453.

Schmitt-Ney M, Habener JF. 2000. CHOP/GADD153 gene expression response to cellular stresses inhibited by prior exposure to ultraviolet light wavelength band C (UVC). Inhibitory sequence mediating the UVC response localized to exon 1. J Biol Chem 275:40839–40845.

Sheehan JP, Palmer PE, Helm GA, Tuttle JB. 1997. MPP^+ induced apoptotic cell death in SH-SY5Y neuroblastoma cells: an electron microscope study. J Neurosci Res 48:226–237.

Tanaka J, Nakamura H, Honda S, Takada K, Kato S. 1988. Neuropathological study on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine of the crab-eating monkey. Acta Neuropathol 75:370–376.

Ubeda M, Habener JF. 2000. CHOP gene expression in response to endoplasmic-reticular stress requires NFY interaction with different domains of a conserved DNA-binding element. Nucleic Acids Res 28: 4987–4997.

Ubeda M, Wang XZ, Zinszner H, Wu I, Habener JF, Ron D. 1996. Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. Mol Cell Biol 16:1479–1489.

Ubeda M, Vallejo M, Habener JF. 1999. CHOP enhancement of gene transcription by interactions with Jun/Fos AP-1 complex proteins. Mol Cell Biol 19:7589–7599.

Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, Boorstein R, Kreibich G, Hendershot LM, Ron D. 1996. Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). Mol Cell Biol 16:4273–4280.

Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D. 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 12:982–995.